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(REV 1-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

3339-422

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

09/530560

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

INTERNATIONAL APPLICATION NO

PCT FR98/02344

INTERNATIONAL FILING DATE

November 3, 1998

PRIORITY DATE CLAIMED

November 3, 1997

TITLE OF INVENTION

TRANSFECTING PEPTIDE VECTOR, COMPOSITION CONTAINING SAME AND THEIR APPLICATIONS

APPLICANT(S) FOR DO/EO/US

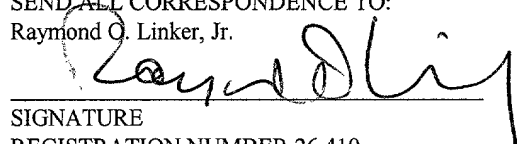
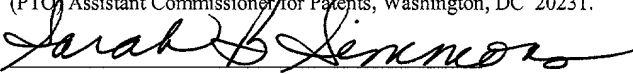
Jadwiga CHROBOCZEK; Pascal FENDER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

## Items 11. To 16. Below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Statement in Support of Filing a Sequence Listing, with descriptive printout;  
Labeled Computer Disk; and  
Copy of amendments made under Article 34 to the French PCT application.

U.S. APPLICATION NO. <b>09 530560</b> (If known, see 37 CFR 1.101)		INTERNATIONAL APPLICATION NO PCT FR/98/02344		ATTORNEY'S DOCKET NUMBER 3339-422			
17. <input checked="" type="checkbox"/> The following fees are submitted:  <b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO <b>\$970.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO <b>\$840.00</b> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search (37 CFR 1.445(a)(2)) paid to USPTO <b>\$690.00</b> International preliminary examination fee (37 CFR 1.482) paid to USPTO <b>\$670.00</b> But all claims did not satisfy provisions of PCT Article 33(1)-(4) <b>\$670.00</b> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) <b>\$ 96.00</b>  <b>ENTER APPROPRIATE BASIC FEE AMOUNT</b> = <b>\$ 840.00</b>				CALCULATIONS		PTO USE ONLY	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$			
CLAIMS		NUMBER FILED		NUMBER EXTRA		RATE	
Total Claims		17 -20 =		0		X \$18.00 \$ 0.00	
Independent Claims		3 - 3 =		0		X \$78.00 \$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ \$260.00		\$ 260.00	
<b>TOTAL OF ABOVE CALCULATIONS</b>				<b>=</b>		<b>\$ 1,100.00</b>	
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).						\$	
<b>SUBTOTAL</b>				<b>=</b>		<b>\$</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).						\$	
<b>TOTAL NATIONAL FEE</b>				<b>=</b>		<b>\$ 1,100.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +						\$	
<b>TOTAL FEES ENCLOSED</b>				<b>=</b>		<b>\$ 1,100.00</b>	
				Amount to be Refunded		\$	
				Charged		\$	
a. <input checked="" type="checkbox"/> A check in the amount of <b>\$1,100.00</b> to cover the above fees is enclosed.							
b. <input type="checkbox"/> Please charge my Deposit Account No. 16-0605 in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.							
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-0605. A duplicate copy of this sheet is enclosed.							
Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.							
SEND ALL CORRESPONDENCE TO: Raymond O. Linker, Jr.  SIGNATURE REGISTRATION NUMBER 26,419 ALSTON & BIRD LLP Post Office Drawer 34009 Charlotte, NC 28234 Tel. Charlotte Office (704) 331-6000 Fax Charlotte Office (704) 334-2014 <b>Customer Number 000826</b>				"Express Mail" Mailing Label Number EL 432837136 US Date of Deposit: May 2, 2000  I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to BOX PCT, Attn: DO/US (PTO) Assistant Commissioner for Patents, Washington, DC 20231.  Sarah B. Simmons			

Attorney Docket No. 3339-422

PATENT

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re: Chroboczek et al. Attn: DO/US  
International Appl. No.: PCT/FR98/02344  
International Filing Date: November 3, 1998  
For: TRANSFECTING PEPTIDE VECTOR,  
COMPOSITION CONTAINING SAME  
AND THEIR APPLICATIONS

May 2, 2000

Box PCT  
Assistant Commissioner of Patents  
Washington, DC 20231

**PRELIMINARY AMENDMENT**

Sir:

Please amend the above-identified application as follows:

In The Specification:

Please add the heading --BACKGROUND OF THE INVENTION-- as Line 3 on Page 1 after the title of the application.

Please add the heading --SUMMARY OF THE INVENTION-- as Line 14 on Page 7 of the application before the paragraph beginning "The subject of...".

Please add the heading --BRIEF DESCRIPTION OF THE DRAWINGS-- as Line 9 on Page 13 of the application before the paragraph beginning "In addition to...".

Please add the heading DETAILED DESCRIPTION AND EXAMPLES as Line 7 on Page 14 of the application before the paragraph beginning "It should be...".

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In The Claims:

3. Transfection vector according to Claim 1 or Claim 2, [characterized in that] wherein the polymeric sequence of the basic amino acids comprises between 10 and 50 amino acid residues, selected from the group consisting of lysine, arginine and ornithine.
4. Transfection vector according to [any one of Claims 1 to 3, characterized in that] claim 1 or 2, wherein the cationic polymeric sequence is selected from the group consisting of polymeric amines.
5. Transfection vector according to [any one of Claims 1 to 4, characterized in that] claim 1 or 2, wherein the NLS sequence is at the N-terminal end of the transfecting peptide and the polymeric sequence of basic amino acids is at the C-terminal end of the said transfecting peptide.
6. Transfection vector according to [any one of Claims 1 to 5, characterized in that] claims 1 or 2, wherein, when the chemical substance is a nucleic acid, the transfecting peptide/nucleic acid ratio is between 0.3:1 and 15:1, preferably between 2:1 and 6:1 [, preferably between 4:1 and 6:1].
7. Transfection vector according to [any one of Claims 1 to 6, characterized in that it is] claims 1 or 2, combined with a targeting ligand.
8. [Composition, characterized in that it essentially consists] A composition consisting essentially of a transfection vector according to [any one of Claims 1 to 7] claim 1 or 2 and a suitable vehicle selected from the group consisting of bile salts, antiproteases, cyclodextrins and derivatives thereof, antiseptics and polyols [, for use as a medicament].
9. [Method] A method of transfecting eukaryotic cells *in vitro* with a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and

pharmacologically active chemical substances, characterized in that it comprises the bringing into contact and the incubation of a transfection vector according to [any one of Claims 1 to 8,] claim 1 or 2 in a dilution buffer comprising 100 – 150 mM NaCl with eukaryotic cells for 15 to 120 minutes at room temperature, the chemical substance to be transfected:transfecting peptide ratio being between 0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

10. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, [characterized in that it contains,] containing, in addition to the said chemical substance, at least one transfecting peptide which comprises:

- a segment of an NLS sequence consisting of sequence ID NO:2,
- a segment of a sequence consisting of sequence ID NO:10,
- a segment of a sequence consisting of sequence ID NO:16, and
- a polylysine [, for use as a medicament].

REMARKS

The above amendments are made to more clearly define the invention under United States practice. Please enter this amendment prior to calculation of the filing fee.

Respectfully submitted,



Raymond O. Linker, Jr.  
Registration No. 26,419

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Sarah B. Simmons

CLT01/4423464v1

TRANSFECTING PEPTIDE VECTOR, COMPOSITION CONTAINING  
SAME AND THEIR APPLICATIONS

The present invention relates to a transfecting  
5 peptide vector, to a composition containing the said  
vector as well as to their applications in the  
treatment (medicaments) and the prevention (vaccines)  
of human and animal diseases. The said vector is in  
particular capable of dispensing to suitable target  
10 cells nucleic sequences, proteins, peptides and  
chemical substances of interest.

In the field of gene therapy, many compositions  
useful for efficiently transfecting eukaryotic cells  
with a selected genetic material have been described.

15 There are essentially two main types of  
transfection vectors:

- the natural transfection vectors, such as  
viruses or modified viruses, which are efficient but  
which have limits to their use: tissue nonspecificity,  
20 necessity to obtain constructs for each gene of  
interest and potential risks for the environment which  
lead to the setting in place of costly and constraining  
clinical infrastructures for the patient and staff;

- nonviral agents (synthetic vectors), capable of  
25 promoting the transfer and the expression of chemical  
substances such as DNA into eukaryotic cells. The  
latter strategy represents an alternative to viral  
vectors.

These synthetic vectors must essentially have  
30 two functions: to condense the DNA to be transfected  
and to promote its cellular attachment as well as its  
passage across the plasma membrane and possibly the  
nuclear membranes; such vectors must therefore mimic  
the functioning of viruses in order to be efficient;  
35 however, it appears that the different vectors provided  
in the prior art do not exhibit these two functions in  
an optimum manner and may, in addition, depending on  
the cases, be toxic for the cells.

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Among these nonviral agents, there may be mentioned first of all the cationic polymers and the cationic lipids. The former generally consist of polylysine, whereas a wide variety of cationic lipids (liposomes or pseudoliposomes) exist, each giving transfection efficiencies which vary according to the cell types (DOTMA, and the like).

The lipid portion which interacts with and/or destabilizes the membranes allows the fusion and the entry of the DNA/liposome complex.

However, the transfection of DNA by liposomes, although less immunogenic than that performed with the aid of cationic polymers, is in fact a relatively inefficient method.

The major mechanism of entry of the DNA/liposome complexes is, it seems, endocytosis; consequently, the transfected DNA is trapped in the intracellular vesicles and destroyed by the lysosomal enzymes.

Even if a portion of the transfected DNA is released into the cytoplasm by a mass action effect, only a small fraction of this DNA is effectively present in the nucleus.

Agents capable of increasing the release of the DNA from the endosomal vesicles and its passage into the nucleus can increase the gene transfer rate.

Among these agents, there may be distinguished:

- those which target the complex towards another point of entry: the targeting is obtained, for example, by coupling ligands with the polylysine polymers; the targeting can also occur after internalization, by directing the complexes to the nucleus (PCT International Application WO 95/31557), and

- those which avoid endosomal degradation; to escape endosomal degradation, it has been proposed to incorporate an endosomolytic agent into the complex, such as adenoviral particles (PCT International

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Application WO 93/07283) or more recently synthetic peptides with endosomolytic activity, which increase the release of the DNA into the cytoplasm.

Taking into account the preceding text, various types of complexes have been provided; there may be mentioned complexes combining liposomes and peptides, such as those described in:

- International Application WO 96/25508, which describes compositions comprising (i) the nucleic acid to be transfected, (ii) a transfection agent, such as a cationic polymer and/or a lipofectant, (iii) a peptide compound involved at the level of the condensation of the nucleic acid, consisting as a whole or in part of peptide motifs possessing a majority of amino acids with a basic character, such as lysine, histidine, arginine (histones, nucleolin, protamine or derivatives thereof) and optionally (iv) a targeting element which makes it possible to orient the transfer of the nucleic acid, such as a ligand of the intracellular type such as a nuclear localization signal sequence (NLS) which favours the accumulation of the transfected DNA inside the nucleus and which may be combined with the peptide compound to form a chimeric peptide comprising a protein fragment (histone or protamine or nucleolin) and an NLS sequence. However, this system requires the presence of a cationic polymer and/or a lipofectant, which have the disadvantage of being toxic and/or costly,

- International Application WO 97/30170 which also describes compositions for transfecting eukaryotic cells, which comprise the nucleic acid to be transfected, at least one cationic lipid at a suboptimal concentration and at least one acidic peptide (active on the membrane) which destabilizes the endosomal membrane and thus increases the transfection efficiency. The positive charge/negative charge ratio is between 0 and 3. The selected peptides are derived from the *influenza* virus, so as to induce effective

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rupture of the endosomes. The presence of the lipids is necessary in this composition because of the fact that the selected peptide does not allow passage across the first cell membrane.

5       Such complexes do not therefore make it possible to avoid the disadvantages linked to the use of liposomes.

That is undoubtedly the reason why complexes using only peptides have been provided:

10       - European Patent Application 0,544,292, which describes a complex for transfecting nucleic acid which comprises a fusion protein consisting of a cellular factor (growth factor, viral antigen, toxin, integrin or lipoprotein) and a basic polycationic peptide  
15       comprising arginine and/or lysine residues,

20       - International Application WO 94/23751, which describes a transfer peptide which comprises three parts: (1) a ligand L1 (peptide of 2 to 100 amino acids), capable of binding to a binding site at the  
25       surface of eukaryotic cells (membrane receptor) (example: peptide RGD, domain for binding of growth factors, hormones, viral antigens or lipoproteins), (2) a ligand L2 similar to L1 (peptide of 2 to 20 amino acids), which binds to the outer nuclear membrane of  
30       eukaryotic cells, such as an NLS sequence and (3) a ligand L3 corresponding to a basic peptide (3 to 100 amino acids) (histone fragment H1 or H2B, for example). The transfer peptides described in this Application therefore have a general ligand structure for a  
35       membrane receptor-ligand for the outer nuclear membrane-basic peptide. Such a structure was proposed in order to improve the specificity of the complex towards target cells, but has a toxicity level of the same order as that of the liposomes; in addition, the construct should be adapted as a function of the target cells (presence of specific receptors on the target cells), and

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- International Application WO 95/31557 which describes a transfection vector comprising a synthetic peptide and the nucleic acid to be transfected. The synthetic peptide comprises a polymeric chain of basic amino acids, preferably at the C-terminal position (10-50 amino acids, such as lysine, arginine and ornithine), an NLS peptide (6-15 amino acids, such as the NLS sequence of the SV40 T antigen, the NLS sequence of the polyoma T antigen, the NLS sequence of adenovirus Ela or the NLS sequence of adenovirus Elb, preferably at the N-terminal position and a hinge region of neutral amino acids (6-50 amino acids selected from glycine, alanine, leucine and isoleucine), between the polymeric chain and the NLS peptide. The preferred NLS sequence is the sequence of the SV40 virus T antigen (small sequence of basic amino acids: PKKKRKV), which is efficient in mammalian cells or a short hydrophobic sequence which contains one or more basic amino acids (KIPIK). The hinge sequence comprises 6-26 neutral amino acids selected solely from Gly (G), Ala (A), Leu (L) and Ile (I). The peptide:DNA ratio (by weight) is between 1:1 and 1:10. The peptide described in this document passes across the cell membrane with difficulty and that is the reason why it is recommended, in this International Application, to treat the cells before the transfection: the cells are treated with a hypertonic solution, and then with a hypotonic solution in the presence of the nucleic acid-peptide complex. The hypertonic solution may contain PEG (0.3 M-0.6 M) and sucrose (10-25%).

These various complexes possess the property of condensing the DNA and of promoting its combination with the cell membrane; however, they are far from being as efficient as the viral vectors, in particular because of an insufficient condensation of the DNA to be transfected and/or of the difficulties encountered by the transfected DNA in coming out of the endosome

without being degraded and in penetrating into the cell nucleus.

Seeking to develop novel vectors not exhibiting the disadvantages of the viral vectors, the inventors developed the vector described in International Application WO 97/18317, which describes compositions comprising an adenoviral protein complex consisting:

A. of an adenoviral protein complex, namely:

- either of 12 pentons, each comprising at least one fibre and one penton base, excluding any other constituent element of the genome of an adenovirus, which fibre(s) and penton base are derived either from the same adenovirus, or from different adenoviruses, the said pentons being linked by the penton bases and forming a dodecahedron structure, stable to proteolytic enzymes, which complex has a molecular weight of between  $4.8 \times 10^6$  and  $6.6 \times 10^6$ ;

- or of 12 penton bases, excluding any other constituent element of the genome of an adenovirus, which penton bases are derived either from the same adenovirus, or from different adenoviruses, and form a dodecahedron structure, stable to proteolytic enzymes and in that it has a molecular weight of between  $3.2 \times 10^6$  and  $4 \times 10^6$ .

B. of a nucleic acid sequence to be transfected, and

C. of a ligand between the adenoviral protein complex and the nucleic acid, such as peptides whose N-terminal portion comprises the N-terminal amino acid sequence of a fibre of an adenovirus of any serotype (region for attachment to the adenoviral protein complex) and whose C-terminal portion comprises a polylysine or a polyarginine.

The transfection vectors described in this Application allow the internalization of the nucleic sequence to be transfected and increase the permeability of the endosomes; this is, however, a relatively complex structure which mimics the behaviour

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of adenoviruses; indeed, the adenoviral particles are relatively complex and comprise several substructures; in particular the outer part or capsid is formed predominantly of three proteins: the hexon, the penton  
5 base and the fibre; the fibre allows the attachment of the virion to a cell receptor, whereas the penton base allows the internalization of the virion.

Continuing their research studies, the inventors have found that, unexpectedly, a peptide  
10 derived from the adenovirus fibre protein is capable of efficiently transfecting nucleic acid sequences or proteins, in the absence of liposomes and of the treatment of cells.

The subject of the present invention is a  
15 peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the said chemical substance,  
20 at least one transfecting peptide derived from the whole or part of an adenovirus fibre and comprising at least one region consisting of at least 50% of hydrophobic amino acids selected from the group consisting of alanine, valine, phenylalanine,  
25 isoleucine, leucine, proline and methionine.

In accordance with the invention, the said peptide is derived from a fibre of an adenovirus selected from the group consisting of Ad2, Ad3, Ad4, Ad7, Ad8, Ad9, Ad11, Ad12, Ad15, Ad16, Ad21, Ad40,  
30 Ad41, FAV1 (CELO) and FAV7 or from a fragment of the SV40 virus Vp3 protein.

According to an advantageous embodiment of the said transfection vector, the said transfecting peptide comprises at least:

35 - a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences: X<sub>0</sub>-Lys-Arg-Val-

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Arg ( $X_0$ KRVR) (SEQ ID NO:1),  $X_0$ -Lys-Arg-Ala-Arg ( $X_0$ KRAR)  
(SEQ ID NO:2),  $X_0$ -Lys-Arg-Ser-Arg ( $X_0$ KRSR) (SEQ ID  
NO:3),  $X_0$ -Lys-Arg-Leu-Arg ( $X_0$ KRLR) (SEQ ID NO:4),  $X_0$ -  
Lys-Arg-Thr-Arg ( $X_0$ KRTR) (SEQ ID NO:5),  $X_0$ -Pro-Lys-Lys-  
5 Pro-Arg ( $X_0$ PKKPR) (SEQ ID NO:6), in which  $X_0$  is zero or  
represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S),  
or a segment of the SV40 virus Vp3 protein and in  
particular the sequence GPNKKKRKL (SEQ ID NO:24),

- a hydrophobic sequence comprising between 7  
10 and 50 amino acids, derived from an adenovirus fibre  
and selected from the group consisting of at least one  
of the following sequences  $X_1$ -Phe-Asn-Pro-Val-Tyr-Pro-  
Tyr- $X_2$  ( $X_1$ FNPVYPY $X_2$ ) (SEQ ID NO:7),  $X_1$ -Phe-Asp-Pro-Val-  
Tyr-Pro-Tyr- $X_2$  ( $X_1$ FDPVYPY $X_2$ ) (SEQ ID NO:8), in which:

15  $X_1$  is zero or represents a sequence of at most  
43 amino acids, preferably a sequence of 5 to 15 amino  
acids, comprising hydrophobic and/or polar and/or  
acidic charged amino acids, and in particular one of  
the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID  
20 NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-  
Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr  
(PSED) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID  
NO:13), Thr-Gln-Tyr-Ala-Glu-Glu-Thr-Glu-Glu-Asn-Asp-Asp  
(TQYAEETEENDD) (SEQ ID NO:14) or  $X_3$ -Glu-Asp-Asp ( $X_3$ EDD)  
25 (SEQ ID NO:15) in which  $X_3$  represents Ala (A), Val (V),  
Leu (L), Phe (F) or Ile (I) and

$X_2$  is zero or represents a sequence of at most  
43 amino acids, preferably a sequence of 5 to 15 amino  
acids, comprising hydrophobic and/or polar and/or  
30 charged amino acids, and in particular one of the  
following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID  
NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-  
Asp-Asn (DADN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD)  
(SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20),  
35 Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser  
(DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID  
NO:23) and

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- a polymeric sequence of basic amino acids or a cationic polymeric sequence or a polyalcohol.

There are understood by:

- hydrophobic amino acids, the following amino acids: Ala, Val, Leu, Ile, Pro, Phe and Met;

- acidic charged amino acids, the following amino acids: Asp and Glu;

- basic charged amino acids, the following amino acids: Lys, Arg and ornithine; and

- neutral polar amino acids, the following amino acids: Gly, Ser, Thr, Cys, Tyr, Asn, Gln, His and Trp.

Advantageously, the said transfecting peptide is branched and comprises at least two fragments which are derived from an adenovirus fibre; the said fragments each comprise a segment of an NLS sequence and a hydrophobic sequence, as defined above and are linked to each other by a polymeric sequence such as a polymeric sequence of basic amino acids.

When the chemical substance is a nucleic acid sequence, it is selected from genes which encode a polypeptide having a therapeutic activity, antisense sequences and ribozymes.

In the case of a coding sequence, it comprises, in addition, an active promoter for the expression of the polypeptide.

The said promoter is in particular selected from the group consisting of constitutive promoters and inducible promoters.

Surprisingly, such a transfecting peptide vector comprising no lipids (in the form of liposomes, for example) or penton base is capable of efficiently transfecting in particular nucleic acid sequences of any size, up to the nucleus and without poisoning the transfected cell.

In all cases, the exogenous nucleic acid sequence, the protein of interest or any other chemical

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substance, combined with the said transfection vector penetrates into the cell (internalization).

Surprisingly, the transfecting peptide-cell receptor interaction significantly increases both the internalization of the transfection vector and the permeability of the endosomes, which significantly increases the passage of the exogenous nucleic acid, of the protein of interest or of any other chemical substance from the endosomes to the cytoplasm and to the nucleus, in comparison with the use of a vector including lipids (in the form of liposomes, for example).

Such transfecting peptide vectors prove surprisingly more efficient and less toxic than compositions containing liposomes (cationic lipids or lipofectants).

According to another advantageous embodiment of the said transfection vector, the said transfecting peptide comprises at least:

- a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences:  $X_0$ -Lys-Arg-Val-Arg ( $X_0$ KRVR) (SEQ ID NO:1),  $X_0$ -Lys-Arg-Ala-Arg ( $X_0$ KRAR) (SEQ ID NO:2),  $X_0$ -Lys-Arg-Ser-Arg ( $X_0$ KRSR) (SEQ ID NO:3),  $X_0$ -Lys-Arg-Leu-Arg ( $X_0$ KRLR) (SEQ ID NO:4),  $X_0$ -Lys-Arg-Thr-Arg ( $X_0$ KRTR) (SEQ ID NO:5),  $X_0$ -Pro-Lys-Lys-Pro-Arg ( $X_0$ PKKPR) (SEQ ID NO:6), in which  $X_0$  is zero or represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S), or a segment of the SV40 virus Vp3 protein and in particular the sequence GPNKKKRKL (SEQ ID NO:24),

- a hydrophobic sequence comprising between 7 and 50 amino acids, derived from an adenovirus fibre and selected from the group consisting of at least one of the following sequences  $X_1$ -Phe-Asn-Pro-Val-Tyr-Pro-Tyr- $X_2$  ( $X_1$ FNPVYPY $X_2$ ) (SEQ ID NO:7),  $X_1$ -Phe-Asp-Pro-Val-Tyr-Pro-Tyr- $X_2$  ( $X_1$ FDPVYPY $X_2$ ) (SEQ ID NO:8), in which:



5  $X_1$  is zero or represents a sequence of at most  
43 amino acids, preferably a sequence of 5 to 15 amino  
acids, comprising hydrophobic and/or polar and/or  
acidic charged amino acids, and in particular one of  
the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID  
NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-  
Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr  
(PSEDT) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID  
NO:13), Thr-Gln-Tyr-Ala-Glu-Glu-Thr-Glu-Glu-Asn-Asp-Asp  
10 (TQYAEETEENDD) (SEQ ID NO:14) or  $X_3$ -Glu-Asp-Asp ( $X_3$ EDD)  
(SEQ ID NO:15) in which  $X_3$  represents Ala (A), Val (V),  
Leu (L), Phe (F) or Ile (I) and

15  $X_2$  is zero or represents a sequence of at most  
43 amino acids, preferably a sequence of 5 to 15 amino  
acids, comprising hydrophobic and/or polar and/or  
charged amino acids, and in particular one of the  
following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID  
NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-  
Asp-Asn (DADN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD)  
20 (SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20),  
Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser  
(DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID  
NO:23), which transfecting peptide is combined with a  
polymeric sequence of basic amino acids, a cationic  
polymer or a polyalcohol.  
25

According to another advantageous embodiment of  
the said transfecting peptide vector, the polymeric  
sequence of basic amino acids comprises between 10 and  
50 amino acid residues, selected from the group  
30 consisting of lysine, arginine and ornithine.

According to another advantageous embodiment of  
the said transfecting peptide vector, the cationic  
polymeric sequence is selected from the group  
consisting of polyamines and quaternary ammonium  
35 polymers; a preferred polyamine is polyethyleneimine  
(PEI).

In accordance with the invention, the said polyalcohol is preferably a C<sub>3</sub>-C<sub>20</sub>, and in particular glycerol or dextrans.

According to another advantageous embodiment of the said transfecting peptide vector, the NLS sequence is at the N-terminal end of the transfecting peptide and the polymeric sequence of basic amino acids is at the C-terminal end of the said transfecting peptide.

According to another advantageous embodiment of the said transfecting peptide vector, when the chemical substance is a nucleic acid, the transfecting peptide/nucleic acid ratio is between 0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

According to another advantageous embodiment of the said transfecting peptide vector, it is combined with a targeting ligand.

The subject of the invention is also a composition, characterized in that it essentially consists of a transfer vector as defined above and a suitable vehicle selected from the group consisting of bile salts, antiproteases, cyclodextrins and derivatives thereof, antiseptics and polyols.

The compositions according to the invention have many applications as medicaments, in human and veterinary medicine:

- in human and animal gene therapy, in particular in hereditary diseases,
- as antiviral agents (antisense sequences or ribozymes),
- as immunogenic or vaccinal agents,
- as antibacterial or anticancer agents, and the like.

The subject of the present invention is also a method of transfecting eukaryotic cells *in vitro* with a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances,

characterized in that it comprises the bringing into contact and the incubation of a transfecting peptide vector in accordance with the invention, in a dilution buffer comprising 100-150 mM NaCl with eukaryotic cells  
5 for 15 to 120 minutes at room temperature, the chemical substance to be transfected:transfecting peptide ratio being between 0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

In addition to the preceding features, the  
10 invention also comprises other features which will emerge from the description which follows, which refers to exemplary embodiments of the method which is the subject of the present invention as well as to the accompanying drawings, in which:

15 - Figure 1 illustrates the transfer of the luciferase gene with peptide I, as a function of time (Figure 1A) or as a function of the NaCl concentration (Figure 1B);

20 - Figure 2 illustrates the kinetics of the expression of the luciferase gene; this figure comprises on the x-axis the transfecting peptide/DNA or DOTAP/DNA ratios and on the y-axis the percentage of transfection at D1 (■), D2 (□), D3 (□) and D6 (□);

25 - Figure 3 represents the migrations obtained on a retardation gel, as a function of the quantity of transfecting peptide;

30 - Figure 4 illustrates the transfections obtained with the liposomes DOSPER and DOTAP; this figure comprises on the x-axis the liposome/DNA ratios and on the y-axis the RLU (Relative Light Unit)/10 sec/10<sup>5</sup> cells;

35 - Figure 5 illustrates the kinetics of entry into the cells of a peptide (peptide I) observed in confocal microscopy; column A shows the fluorescent peptide and column B shows the cellular nucleic acids stained with propidium iodide;

- Figure 6 illustrates the inhibition of transfection with peptide I, after preincubation with

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an excess of peptide I. The HeLa cells in plates comprising 24 wells are preincubated with peptide I for one hour at 4°C at concentrations of 10 to 50 µg/ml, respectively;

5               - Figure 7 represents a few sequences of adenovirus fibres.

It should be clearly understood, however, that these examples are given solely by way of illustration of the subject of the invention and do not constitute  
10 in any manner a limitation thereof.

EXAMPLE 1: Materials and methods

- Cells, plasmid and peptides:

The HeLa cells are cultured at 37°C in an EMEM  
15 medium supplemented with 10% foetal calf serum under an atmosphere containing 5% CO<sub>2</sub>.

A luciferase reporter vector (plasmid pGL3, Promega) is used to demonstrate the transfection.

The peptide IC comprises sequence No. 2,  
20 sequence No. 10, sequence No. 7, sequence No. 16, from the N-terminal end to the C-terminal end and 10 lysines; this peptide corresponds to the 20 N-terminal residues of the Ad3 fibre. Peptide I contains these same 20 N-terminal amino acids of the Ad3 fibre and 20  
25 lysines; the peptides were obtained by solid-phase synthesis, followed by HPLC purification.

This peptide I is labelled with fluorescein; peptides comprising 10 lysines instead of 20 were also prepared. The integrity of all the peptides is verified  
30 by mass spectroscopy.

- Retardation gel:

500 ng of plasmid DNA (pGL3) are preincubated with various quantities of peptides for 5 min at room temperature, and then subjected to electrophoresis on a  
35 1% agarose gel prepared in a TBE buffer.

After electrophoresis at 50 V in the TBE buffer for 30 min, the DNA on the gel being stained with

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ethidium bromide and visualized under ultraviolet light.

- Transfections:

1.5 to 12 µg of peptide I in 50 µl of dilution  
5 buffer (20 mM Tris, pH 7.4, 150 mM NaCl) are incubated  
with 1.5 µg of plasmid pGL3 in 250 µl of EMEM medium  
for 15 to 30 min at room temperature.

The mixture of DOTAP or of DOSPER with 1.5 µg  
of plasmid pGL3 is prepared according to the  
10 manufacturer's instructions (Boehringer). For the  
studies of the effect of the peptide on transfection in  
the presence of liposomes, portions of peptides are  
mixed with DOTAP or DOSPER in the dilution buffer  
defined above for 15 to 30 min at room temperature, and  
15 then 1.5 µg of plasmid pGL3 are added and incubated for  
15 min at room temperature.

The transfections are carried out in plates  
comprising 24 wells (Beckton Dickinson) with  $1.5 \times 10^5$   
cells/well (confluence of about 50%) for 1 h at 37°C.  
20 After 24 h, the light emission is measured in the cell  
lysates with the Promega Luciferase Assay System test.

- Haemolytic test:

Human erythrocytes are washed 3 times with PBS  
buffer.  $10^6$  erythrocytes are incubated with 6 µg of  
25 peptide I for various periods of time at 37°C. After  
centrifugation at 10,000 g for 5 min, the optical  
density of the supernatant is measured at 540 nm.

- Internalization of the peptide:

HeLa cells, cultured on cover slips ( $10^5$   
30 cells/cover slip) are treated with 3% bovine serum  
albumin in EMEM for 15 min at 37°C.

The cells are washed twice with PBS buffer,  
incubated with 40 µg/ml of peptide I labelled with  
fluorescein for various durations at 37°C, fixed with  
35 2% paraformaldehyde in PBS for 20 min at 37°C, washed  
with PBS and stained with 1 µg/ml of propidium iodide  
in PBS for 5 min at room temperature.

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The cover slips are mounted on a microscope slide with 1,4-diazabicyclooctane (Sigma) and observed under an MRC600 confocal microscope (BioRad).

\* Results:

5       - Cell transfection with peptide I:

All the experiments were carried out with HeLa cells transfected with the plasmid pGL3 (Stratagene) carrying the luciferase gene. For peptide I/DNA ratios equal to 2, the optimum time for interaction between  
10 the transfecting complexes and the cells is between 60 and 120 min (Figure 1A).

The effect of the NaCl concentrations was tested for 1 h of transfection on gene expression measured 24 h after transfection (Figure 1B).

15       A transfection optimum exists for peptide I/DNA ratios of between 4 and 6 and for an NaCl concentration of 125 mM. Concentrations of less than 100 mM and greater than 150 mM appear to be inhibitory.

The expression of the transgene may be observed  
20 up to 6 days after transfection (Figure 2). However, the addition of 2% serum completely abolishes the transfection with peptide I.

The behaviour of the DNA/peptide complexes is analysed on retardation gels. In theory, 526 peptide  
25 molecules are necessary to neutralize the phosphate charges carried by the plasmid (5256 bp), which means that 322 ng of peptide I are necessary for complete neutralization of 500 ng of plasmid.

Figure 3 shows that incubation with 250 ng of  
30 peptide causes retardation in the migration of the DNA and the addition of 500 ng of peptide completely stops its migration, which is in agreement with the theoretical considerations set out above.

The highest transfection efficiency is observed  
35 when an excess of neutralizing peptide charges relative to the DNA of the order of 4 exists (Figure 1B), which confirms that the transfer of the gene occurs only in the presence of an excess of positive charges.

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- Parameters involved in the efficiency of transfection with the peptide according to the invention:

5       The peptides according to the invention  
comprise essentially 3 domains: the nuclear  
localization signal, the hydrophobic domain and the  
basic polymer.

10       To study the effect of the structure of the  
peptide on the transfection of DNA, a series of  
peptides [lacuna], in which the various portions of  
peptide I have been removed, whose sequences are  
illustrated in Table I below:

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TABLE I

Peptide	Sequences*
I	A K R A R L S T S F N P V Y P Y E D E S - K <sub>20</sub> = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16-K <sub>20</sub>
IC	A K R A R L S T S F N P V Y P Y E D E S - K <sub>10</sub> = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16-K <sub>10</sub>
IE	A K R A R L S T S E D E S-K <sub>10</sub> = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:16- K <sub>10</sub>
ID	L S T S F N P V Y P Y E D E S-K <sub>20</sub> = SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16- K <sub>20</sub>
IA	A K R A R L S T S F N P V Y P Y E D E S = SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16
LII	A K R A R L S T S F N P V Y P Y E D E S A K R A R L S T S F N P V Y P Y E D E S (for each branch: SEQ ID NO:2 + SEQ ID NO:10 + SEQ ID NO:7 + SEQ ID NO:16)

\* in which X<sub>0</sub> = A.

5           The results obtained with these various  
peptides are illustrated in Table II below.



TABLE II

Peptide ( $\mu\text{g}$ )	DNA ( $\mu\text{g}$ )	RLU/10 s/10 <sup>5</sup> cells x 10 <sup>3</sup>
<u>Peptide I</u>		
3	1.5	450
6	1.5	4920
9	1.5	1450
12	1.5	1170
<u>Peptide IC</u>		
3.6	1.5	1
9	1.5	2870
12	1.5	1330
<u>Peptide IE</u>		
3	1.5	1
6	1.5	10
9	1.5	110
12	1.5	330
<u>Peptide ID</u>		
3-12	1.5	1
<u>Peptide IA</u>		
3-12	1.5	1
<u>Peptide K<sub>10</sub></u>		
3-12	1.5	1
<u>Peptide K<sub>20</sub></u>		
3-12	1.5	1-10

The results presented in Table II show that the transfection efficiency depends on the presence of the nuclear localization signal sequence of the adenovirus fibre protein.

There is no transfection when the NLS domain (peptide ID), polylysine (peptide IA) or both the NLS domain and the hydrophobic region (peptide K<sub>10</sub> and peptide K<sub>20</sub>) are removed. The removal of the hydrophobic domain between the NLS portion and the polylysine portion induces a significant effect: this peptide (peptide IE) is approximately 30 times less efficient than peptide IC. Even if peptide ID is still capable of

attaching and condensing the DNA and of entering into the cell, it appears nevertheless incapable of transporting this DNA into the nucleus.

5 It is also evident from these results that when the number of lysines present in the polylysine polymer (peptide IC) is reduced, a modest negative effect is observed on the transfection efficiency which can be compensated for by increasing the quantity of peptide necessary for an efficient transfer.

10 Table III below illustrates other results obtained with peptide I or peptide IA, in the presence of glycerol or of PEI and peptide LII.

TABLE III

15

Peptide (or PEI) ( $\mu$ g)	DNA ( $\mu$ g)	RLU/10 s/10 <sup>5</sup> cells x 10 <sup>3</sup>
<u>Peptide I</u>		
6	1.5	12,600
6 (+ 10% glycerol)	1.5	34,900
6 (+ 0.1% DMSO)	1.5	11,320
<u>Peptide LII</u>		
1.5	1.5	0
3	1.5	5200
6	1.5	2400
<u>PEI</u>		
3	1.5	66,400
4	1.5	51,200
<u>Peptide IA + PEI</u>		
1.2 $\mu$ g	1.5	104,000
1.6 $\mu$ g	1.5	84,800

The peptide vector comprising peptide I and PEI (covalent complex) is more efficient than PEI (polyethyleneimine) alone.

20 The transfection efficiency is significantly greater in the case of the covalent complex.

It can be said that 1 µg of peptide I/PEI gives 54,000-67,000 RLU, while 1 µg of PEI gives 13,000-22,000 RLU.

5     - Effect of the peptide on transfection in the presence of liposomes:

Two liposomes were used, the liposome DOTAP and the liposome DOSPER, both marketed by Boehringer.

They consist of cationic lipids with internal ester bonds capable of being degraded by cellular  
10   lipases or esterases, which ought to confer on these liposomes a cytotoxicity lower than that observed with other liposomes.

The conditions for transfecting the HeLa cells were optimized for the liposomes alone (Figure 4). The  
15   transfection with the liposome DOTAP leads to lower efficiencies than those observed with the liposome DOSPER; however, the transfections with the liposome DOTAP tend to show a plateau above a certain DNA/liposome ratio, whereas the transfections with the  
20   liposome DOSPER show a peak. For the simultaneous peptide/liposome transfections, the order in which these compounds are added is important, since a higher efficiency (increase of several factors) is observed when the liposome is first mixed with the peptide (and  
25   not with the DNA) and then when the plasmid DNA is added after 15 min of incubation at room temperature.

Table IV below illustrates the results obtained.

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TABLE IV

RLU/10 sec/10<sup>5</sup> cells x 10<sup>3</sup>

DOTAP:DNA no peptide I		Peptide I:DNA	
		2	4
2	9320 ± 820	28,900 ± 890	30,440 ± 2360
4	18,590 ± 570	15,050 ± 340	17,750 ± 750
6	4140 ± 640	3800 ± 770	7300 ± 150
DOSPER:DNA no peptide I		Peptide I:DNA	
		1	2
4	822 ± 78		4
5.5	6880	3160 ± 760	11,102 ± 2370
		18,820	13,760
			12,840
No liposome		13,550 ± 2930	35,500 ± 4800

The results illustrated in this Table IV were obtained under the following conditions:

portions of the peptide I are mixed with 1.5 µg of plasmid pGL3 in 300 µl of dilution buffer for 15 min at room temperature.

The liposome DOTAP is mixed with 1.5 µg of plasmid pGL3 in a DOTAP/DNA ratio of 2 or 4 (v/w), in 300 µl of dilution buffer for 15 min at room temperature.

Surprisingly, these results show that in the absence of liposomes, the results observed with the peptide alone in peptide/DNA ratios of 4 are superior to those observed with the liposome/peptide mixture.

- Intracellular location of the peptide:

The cellular distribution of the fluorescent peptide I is monitored by confocal microscopy (Figure 5).

The first observations, carried out 2 min after the transfection, show a certain quantity of signal at the periphery of the cell.

5 to 10 min after the addition of the peptide, a strong cytoplasmic signal is observed, indicating the entry of the peptide into the cell.

At 30 min, the signal is also observed in the nucleus and a very bright signal is observed in the nucleoli. This substantial transfer into the nucleoli is particularly surprising.

The observations made between 60 and 120 min show the passage of the peptide again into the cytoplasm and at the periphery of the cell until the signal is completely lost.

The results illustrated in this Figure 4 were obtained with peptide concentrations twice as high (40 µg/ml) as those used for the DNA transfection assays. When the usual concentrations for the transfections are used, the accumulation of peptides is slower, but follows the same steps as those specified above.

- Mechanism of the internalization of the peptide:

A haemolytic test carried out with peptide I on erythrocytes gives negative results which show that the interaction of the cell with peptide I is not linked to the formation of pores. Since no expression of luciferase is observed when the transfections are carried out at 4°C, it appears that the mechanism of internalization of the peptide depends on endocytosis and involves the cytoskeleton.

To determine if sites for specific attachment of peptide I to the plasma membrane exist, cells were preincubated with peptide I at 4°C for 2 h, so as to try to saturate the possible sites of attachment of the peptide.

This resulted in complete inhibition of transfection (Figure 6), which indicates that the latter involves a receptor-dependent endocytosis.

- Size of the transfecting complex:

Light scattering can be used to measure the size of the complexes and their distribution, if a mixed population is formed. Under these conditions, it is possible to study the effect of the incubation time on the formation of the transfecting complexes.

The size is measured immediately after the mixing and 1 h later. The complexes prepared by mixing the liposome DOTAP with a DNA plasmid have a diameter of about 115 nm and their size does not change during the incubation.

The DNA/peptide I complexes are larger and have a diameter of about 350-360 nm. Furthermore, the formation of the complex is a dynamic process since a rapid increase in size is observed as a function of the incubation time when more than 90% of the complexes reach a diameter of 660-1100 nm after 1 h.

The size and the distribution of the complexes with peptide I is similar regardless of the peptide/DNA ratio (from 1 to 8).

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The effect of the size of the complex on the transfection efficiency was studied using complexes prepared by varying the period of incubation at room temperature. The results are illustrated in Table V below.

TABLE V

RLU/10 sec/10 <sup>5</sup> cells x 10 <sup>3</sup>	
Peptide:DNA	Preincubation time at room temperature (min)
<u>Experiment 1:</u>	15 60 120
2	410 150 827
4	2540 1900 1460
6	702 3460 980
<u>Experiment 2:</u>	0 15 30 60
4	400 3240 4950 4120
<u>Experiment 3:</u>	0 15 30 60 120
4	1710 14,550 23,170 32,175 6800

Surprisingly, with the complexes according to the invention, even very large aggregates can be transfected.

To measure the size of the transfected substance, light scattering measurements were carried out with an ion-argon laser (Spectra Physics 1161) at 488 nm and 150 mW (geometry of scattering at 90°). The spectrum is accumulated for 200 s using a Malvern 7032 correlator (Malvern Instruments) and then repeated 1 h later. All the spectra are in homodyne mode: the amplitudes of the intensity correlation function with a zero retardation are consistent with the special coherence factor  $\beta$  obtained with a suspension of dilute latex, namely  $\beta = 0.90$ .

The hydrodynamic rays  $R_H$  are calculated using the Malvern multimodal procedure (Pike-Ostrowsky), in order to characterize the principal rates of degradation of the field correlation function with the Stokes-Einstein equation  $R_H = k_s T Q^2 / (6\pi\eta\Gamma_1)$ , in which  $\Gamma_1$

is the principal degradation rate,  $T$  is the absolute temperature of the thermal bath (298 K),  $Q$  the transfer wave vector and  $\eta$  the viscosity of the solvent.

As is evident from the above, the invention is  
5 not at all limited to its embodiments, methods of  
implementation and methods of application which have  
just been described more explicitly; it embraces, on  
the contrary, all the variants which may occur to a  
specialist in this field, without departing from the  
10 framework or scope of the present invention.

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CLAIMS

1. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the said chemical substance, at least one transfecting peptide derived from the whole or part of a fibre of an adenovirus selected from the group consisting of Ad2, Ad3, Ad4, Ad7, Ad8, Ad9, Ad11, Ad12, Ad15, Ad16, Ad21, Ad40, Ad41, FAV1 (CELO) and FAV7, which transfecting peptide comprises at least:

- a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences: X<sub>0</sub>-Lys-Arg-Val-Arg (X<sub>0</sub>KRVR) (SEQ ID NO:1), X<sub>0</sub>-Lys-Arg-Ala-Arg (X<sub>0</sub>KRAR) (SEQ ID NO:2), X<sub>0</sub>-Lys-Arg-Ser-Arg (X<sub>0</sub>KRSR) (SEQ ID NO:3), X<sub>0</sub>-Lys-Arg-Leu-Arg (X<sub>0</sub>KRLR) (SEQ ID NO:4), X<sub>0</sub>-Lys-Arg-Thr-Arg (X<sub>0</sub>KRTR) (SEQ ID NO:5), X<sub>0</sub>-Pro-Lys-Lys-Pro-Arg (X<sub>0</sub>PKKPR) (SEQ ID NO:6), in which X<sub>0</sub> is zero or represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S), or a segment of the SV40 virus Vp3 protein and in particular the sequence GPNKKKRKL (SEQ ID NO:24),

- a hydrophobic sequence comprising between 7 and 50 amino acids, derived from an adenovirus fibre and selected from the group consisting of at least one of the following sequences X<sub>1</sub>-Phe-Asn-Pro-Val-Tyr-Pro-Tyr-X<sub>2</sub> (X<sub>1</sub>FNPVYPYX<sub>2</sub>) (SEQ ID NO:7), X<sub>1</sub>-Phe-Asp-Pro-Val-Tyr-Pro-Tyr-X<sub>2</sub> (X<sub>1</sub>FDPVYPYX<sub>2</sub>) (SEQ ID NO:8), in which:

X<sub>1</sub> is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or acidic charged amino acids, and in particular one of the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr

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(PSED) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID NO:13), Thr-Gln-Tyr-Ala-Glu-Glu-Thr-Glu-Glu-Asn-Asp-Asp (TQYAEETEENDD) (SEQ ID NO:14) or X<sub>3</sub>-Glu-Asp-Asp (X<sub>3</sub>EDD) (SEQ ID NO:15) in which X<sub>3</sub> represents Ala (A), Val (V),  
5 Leu (L), Phe (F) or Ile (I) and

X<sub>2</sub> is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or charged amino acids, and in particular one of the  
10 following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-Asp-Asn (DADN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD) (SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20), Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser  
15 (DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID NO:23) and

- a polymeric sequence of basic amino acids or a cationic polymeric sequence or a polyalcohol, for use as a medicament.

20 2. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the  
25 said chemical substance, at least one transfecting peptide derived from the whole or part of a fibre of an adenovirus selected from the group consisting of Ad2, Ad3, Ad4, Ad7, Ad8, Ad9, Ad11, Ad12, Ad15, Ad16, Ad21, Ad40, Ad41, FAV1 (CELO) and FAV7, which transfecting  
30 peptide comprises at least:

- a segment of an NLS sequence derived from an adenovirus fibre comprising between 4 and 5 amino acids and including a sequence selected from the group consisting of the following sequences: X<sub>0</sub>-Lys-Arg-Val-Arg (X<sub>0</sub>KRVR) (SEQ ID NO:1), X<sub>0</sub>-Lys-Arg-Ala-Arg (X<sub>0</sub>KRAR) (SEQ ID NO:2), X<sub>0</sub>-Lys-Arg-Ser-Arg (X<sub>0</sub>KRSR) (SEQ ID NO:3), X<sub>0</sub>-Lys-Arg-Leu-Arg (X<sub>0</sub>KRLR) (SEQ ID NO:4), X<sub>0</sub>-Lys-Arg-Thr-Arg (X<sub>0</sub>KRTR) (SEQ ID NO:5), X<sub>0</sub>-Pro-Lys-Lys-  
35

Pro-Arg ( $X_0$ PKKPR) (SEQ ID NO:6), in which  $X_0$  is zero or represents Thr (T), Ala (A), Ser-Lys (SK) or Ser (S), or a segment of the SV40 virus Vp3 protein and in particular the sequence GPNKKKRKL (SEQ ID NO:24),

- 5           - a hydrophobic sequence comprising between 7 and 50 amino acids, derived from an adenovirus fibre and selected from the group consisting of at least one of the following sequences  $X_1$ -Phe-Asn-Pro-Val-Tyr-Pro-Tyr- $X_2$  ( $X_1$ FNPVYPY $X_2$ ) (SEQ ID NO:7),  $X_1$ -Phe-Asp-Pro-Val-Tyr-Pro-Tyr- $X_2$  ( $X_1$ FDPVYPY $X_2$ ) (SEQ ID NO:8), in which:

- 10            $X_1$  is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or acidic charged amino acids, and in particular one of the following sequences: Leu-Ser-Asp-Ser (LSDS) (SEQ ID NO:9), Leu-Ser-Thr-Ser (LSTS) (SEQ ID NO:10), Leu-Ser-Ser-Ser (LSSS) (SEQ ID NO:11), Pro-Ser-Glu-Asp-Thr (PSED) (SEQ ID NO:12), Val-Asp-Asp-Gly (VDDG) (SEQ ID NO:13), Thr-Gln-Tyr-Ala-Glu-Glu-Thr-Glu-Glu-Asn-Asp-Asp (TQYAEETEENDD) (SEQ ID NO:14) or  $X_3$ -Glu-Asp-Asp ( $X_3$ EDD) (SEQ ID NO:15) in which  $X_3$  represents Ala (A), Val (V), Leu (L), Phe (F) or Ile (I) and

- 20            $X_2$  is zero or represents a sequence of at most 43 amino acids, preferably a sequence of 5 to 15 amino acids, comprising hydrophobic and/or polar and/or charged amino acids, and in particular one of the following sequences: Glu-Asp-Glu-Ser (EDES) (SEQ ID NO:16), Asp-Thr-Glu-Thr (DTET) (SEQ ID NO:17), Asp-Ala-Asp-Asn (DADN) (SEQ ID NO:18), Asp-Pro-Phe-Asp (DPFD) (SEQ ID NO:19), Gly-Tyr-Ala-Arg (GYAR) (SEQ ID NO:20), Glu-His-Tyr-Asn (EHYN) (SEQ ID NO:21), Asp-Thr-Ser-Ser (DTSS) (SEQ ID NO:22) or Asp-Thr-Phe-Ser (DTFS) (SEQ ID NO:23), which transfecting peptide is combined with a polymeric sequence of basic amino acids, a cationic polymer or a polyalcohol,
- 35           for use as a medicament.

3.           Transfection vector according to Claim 1 or Claim 2, characterized in that the polymeric sequence

of basic amino acids comprises between 10 and 50 amino acid residues, selected from the group consisting of lysine, arginine and ornithine.

4. Transfection vector according to any one of Claims 1 to 3, characterized in that the cationic polymeric sequence is selected from the group consisting of polymeric amines.

5. Transfection vector according to any one of Claims 1 to 4, characterized in that the NLS sequence is at the N-terminal end of the transfecting peptide and the polymeric sequence of basic amino acids is at the C-terminal end of the said transfecting peptide.

6. Transfection vector according to any one of Claims 1 to 5, characterized in that when the chemical substance is a nucleic acid, the transfecting peptide/nucleic acid ratio is between 0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

7. Transfection vector according to any one of Claims 1 to 6, characterized in that it is combined with a targeting ligand.

8. Composition, characterized in that it essentially consists of a transfection vector according to any one of Claims 1 to 7 and a suitable vehicle selected from the group consisting of bile salts, antiproteases, cyclodextrins and derivatives thereof, antiseptics and polyols, for use as a medicament.

9. Method of transfecting eukaryotic cells in vitro with a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it comprises the bringing into contact and the incubation of a transfection vector according to any one of Claims 1 to 8, in a dilution buffer comprising 100-150 mM NaCl with eukaryotic cells for 15 to 120 minutes at room temperature, the chemical substance to be transfected:transfecting peptide ratio being between

0.3:1 and 15:1, preferably between 2:1 and 6:1, preferably between 4:1 and 6:1.

10. Peptide vector for transfecting a chemical substance selected from the group consisting of nucleic acid sequences, proteins, peptides and pharmacologically active chemical substances, characterized in that it contains, in addition to the said chemical substance, at least one transfecting peptide which comprises:

- 10 - a segment of an NLS sequence consisting of sequence ID NO:2,
  - a segment of a sequence consisting of sequence ID NO:10,
  - a segment of a sequence consisting of
  - 15 sequence ID NO:16, and
  - a polylysine,
- for use as a medicament.

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## LEGEND TO THE FIGURES

## Legend to Figure 1

1. Time (min)
- 5 2. Peptide:DNA ratio

## Legend to Figure 2

1. Percentage of transfection
- 10 2. Peptide/DNA
3. DOTAP/DNA
4. Day

## 15 Legend to Figure 4

1. RLU/10 sec/ $10^5$  cells
2. Liposome/DNA ratio

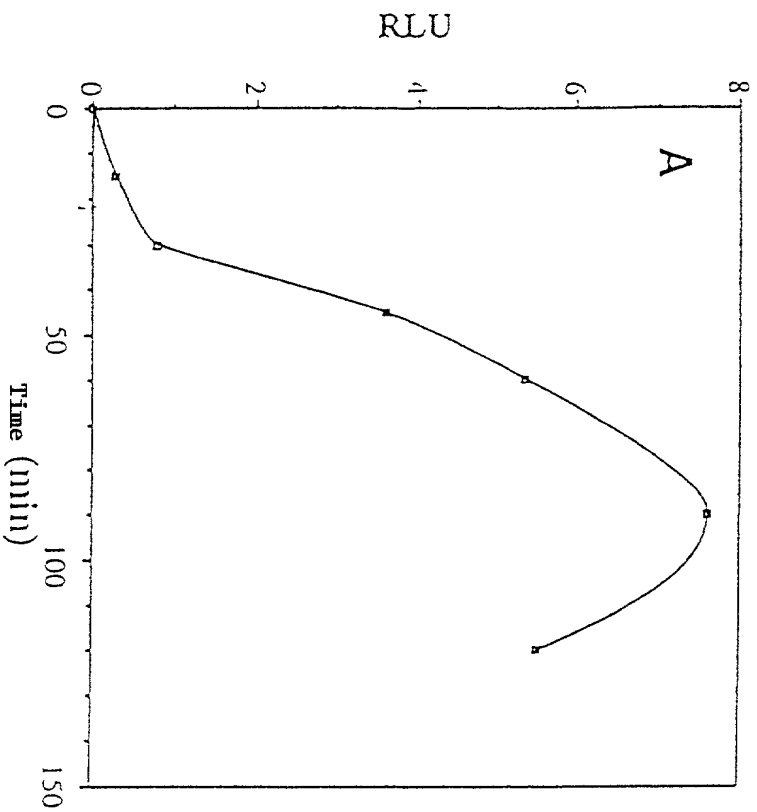
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## Legend to Figure 6

1. Peptide I/DNA ratio
2. Control

05530560.051900

Peptide



Peptide/NaCl

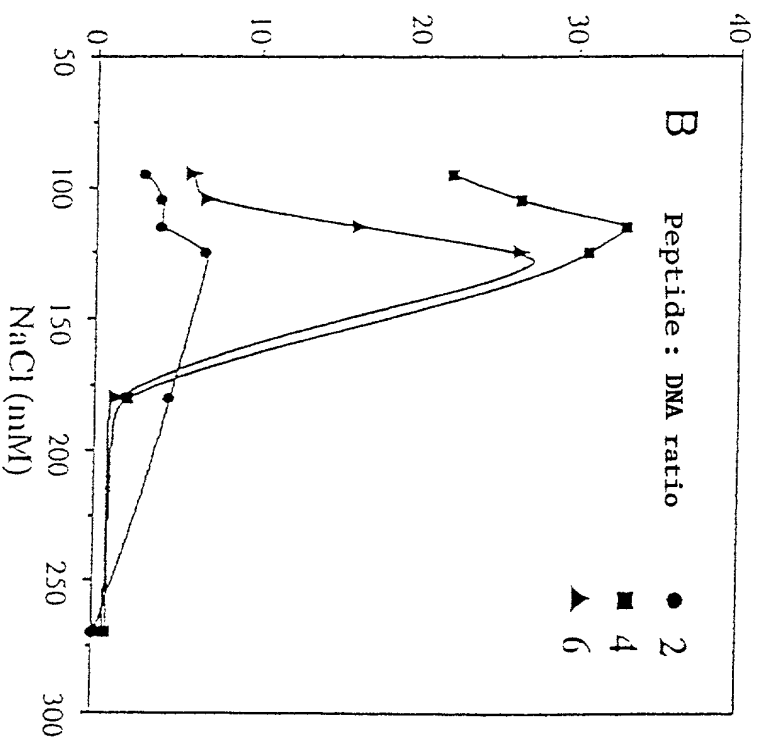


FIGURE 1

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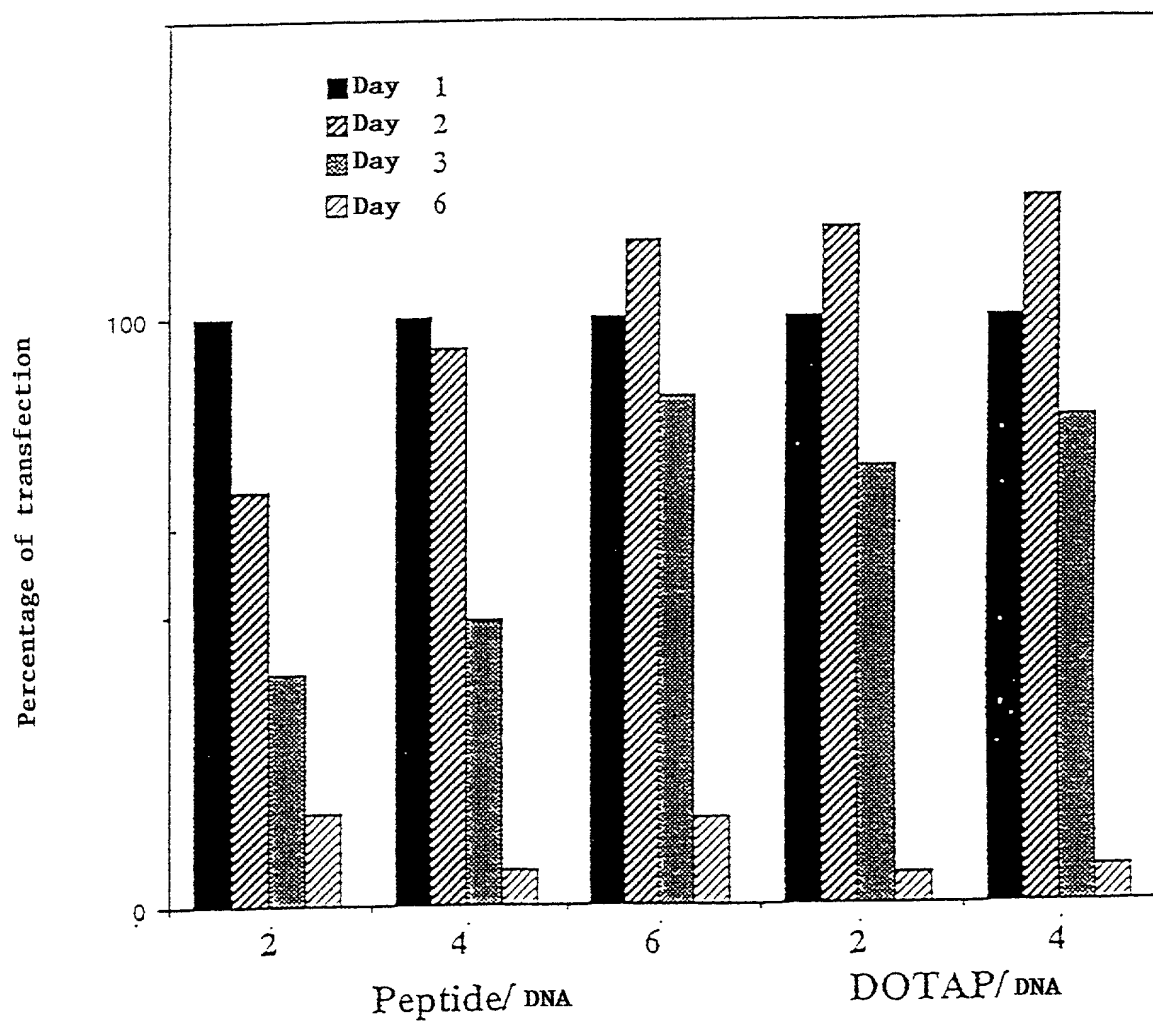


FIGURE 2



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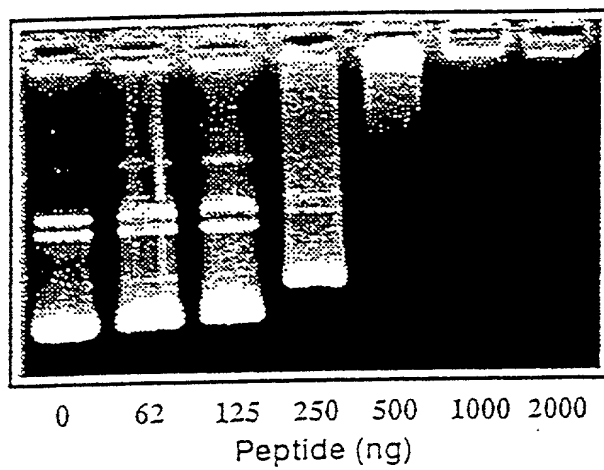


FIGURE 3

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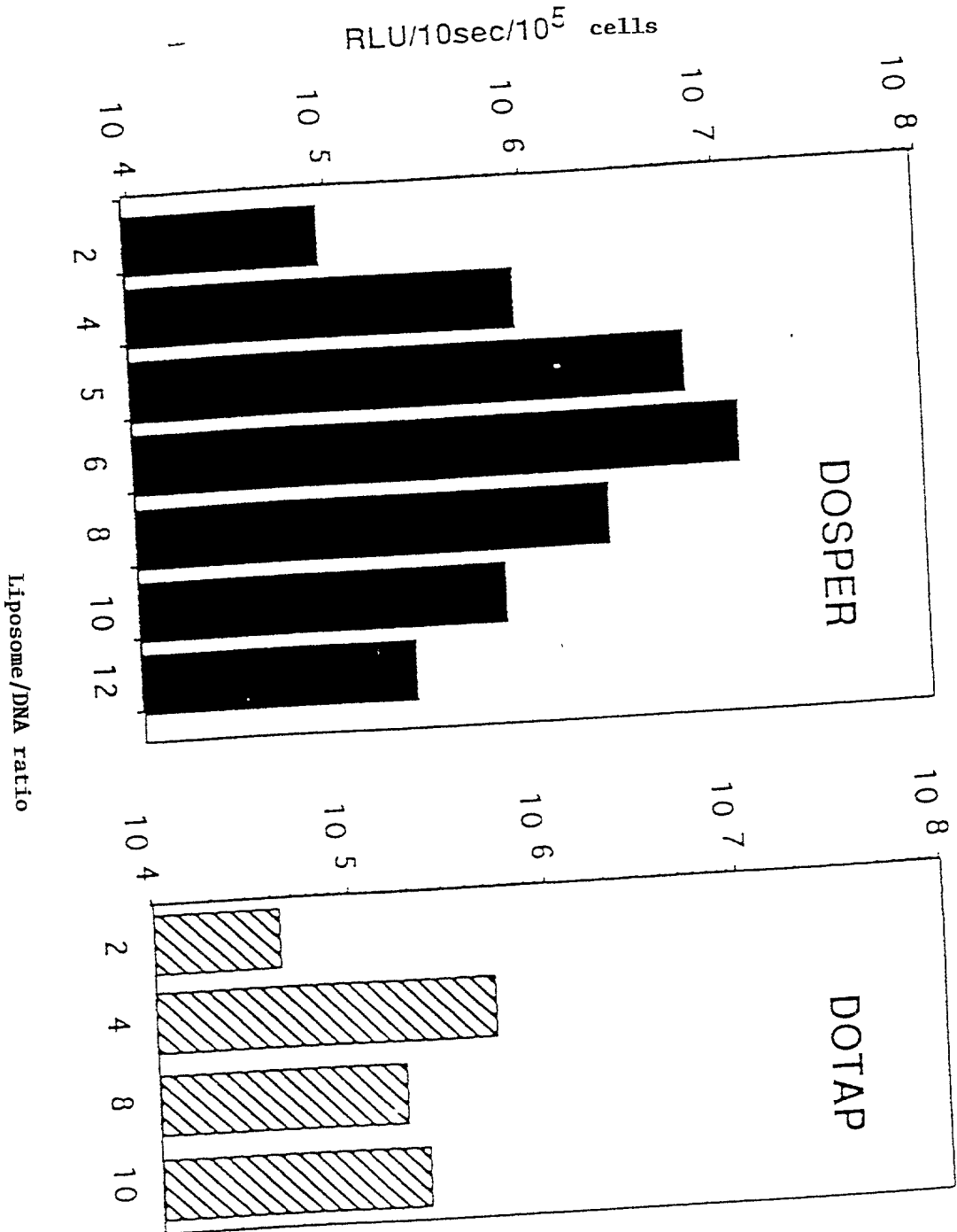


FIGURE 4

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WO 99/23237

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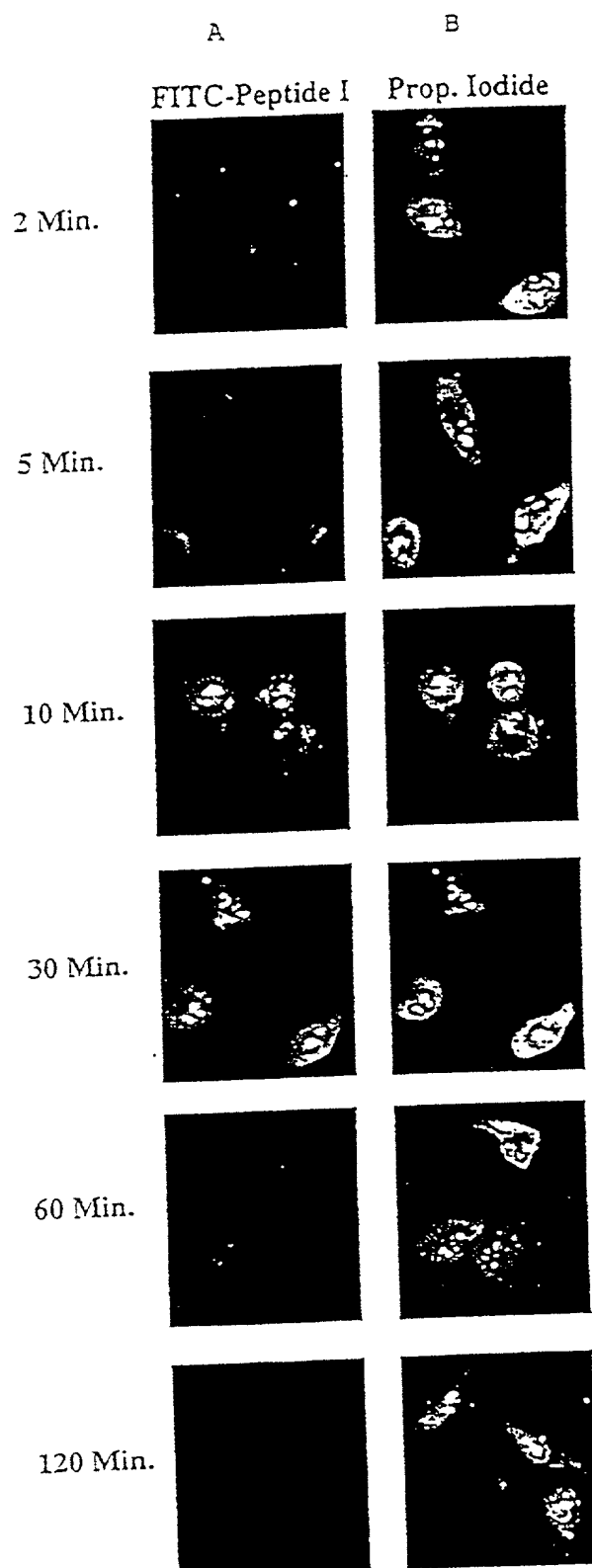
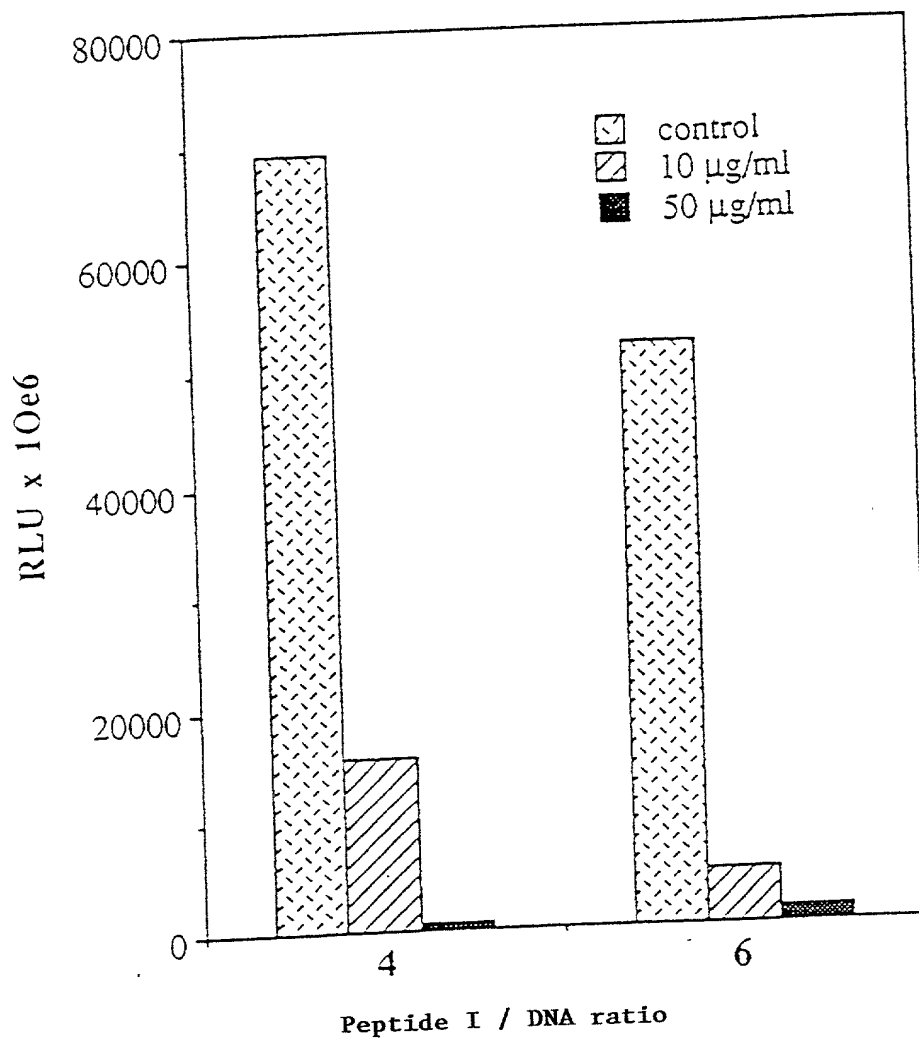


FIGURE 5

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FIGURE 6

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AD7	1	MT	-	KRVRLS	-	-	-	-	-	DS	FNPVYPY	EDESYSQ	-	HPFINPGF
AD21	1	MT	-	KRVRLS	-	-	-	-	-	DS	FNPVYPY	EDESYSQ	-	HPFINPGF
AD3	1	MA	-	KRARL	S	-	-	-	-	TS	FNPVYPY	EDESYSQ	-	HPFINPGF
AD16	1	MA	-	KRARL	S	-	-	-	-	SS	FNPVYPY	EDESYSQ	-	HPFINPGF
AD2	1	M	-	KRARPS	-	-	-	-	-	ED	T FNPVYPY	DETGPPT	-	VPFLTTPPF
AD5	1	M	-	KRARPS	-	-	-	-	-	ED	T FNPVYPY	DETGPPT	-	VPFLTTPPF
AD4	1	MSK	-	KRARV	-	-	-	-	-	DD	GFDPVYPY	DADNA	PT	VPFINPPF
AD12	1	M	-	KRSRTQYAEETE	-	-	-	-	-	DD	FNPVYPY	DPFDTSD	-	VPFVTPPF
AD8	1	MT	-	KRLRA	-	-	-	-	-	ED	FNPVYPY	GYARNQN	-	IPFLTTPPF
AD9	1	MS	-	KRLRV	-	-	-	-	-	ED	FNPVYPY	GYARNQN	-	IPFLTTPPF
AD15	1	MS	-	KRLRV	-	-	-	-	-	ED	FNPVYPY	GYARNQN	-	IPFLTTPPF
AD41-1	1	M	-	KRARL	-	-	-	-	-	ED	FNPVYPY	EHYN	PLD	IPFITPPF
AD40-1	1	M	-	KRARF	-	-	-	-	-	ED	FNPVYPY	EHYN	PLD	IPFITPPF
AD40-2	1	M	-	KRTRI	-	-	-	-	-	ED	FNPVYPY	DTSS	TPS	IPYVAPPF
AD41-2	1	M	-	KRTRI	-	-	-	-	-	ED	FNPVYPY	DTFS	TPS	IPYVAPPF

FIGURE 7

# DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(Foreign Agent Involved)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.  
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled  
Transfecting peptide vector, composition containing same and their applications

the specification of which is attached hereto unless the following box is checked:

[ ] was filed on \_\_\_\_\_ as United States Application Number or PCT International Application Number \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

## Prior Foreign Application(s)

## Priority Claimed

<u>PCT/FR98/02344</u>	<u>FRANCE</u>	<u>3 November 1998</u>	<input checked="" type="checkbox"/> [ ]	<input type="checkbox"/> [ ]
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
<u>97 13771</u>	<u>FRANCE</u>	<u>3 November 1997</u>	<input checked="" type="checkbox"/> [ X ]	<input type="checkbox"/> [ ]
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefits under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____	_____
(Number)	(Filing Date)
_____	_____
(Number)	(Filing Date)

006750 09508550

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C., § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Appln. Serial No.) (Filing Date) (Status --patented/pending/aban.)

(Appln. Serial No.) (Filing Date) (Status --patented/pending/aban.)

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from my French representatives, Cabinet Ores, as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

**Customer Number 826**

ADDRESS CORRESPONDENCE TO THE ATTENTION OF: Raymond O. Linker, Jr. Registration No. 26,419	DIRECT ALL TELEPHONE CALLS TO: Raymond O. Linker, Jr. Registration No. 26,419 Tel (704) 331-6000 Fax (704) 334-2014
---	---

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's Signature: J. Chroboczek Date: 4/24/2000

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Full name of second inventor: <sup>2-00</sup> Pascal FENDER

Inventor's Signature: 

Date: 26/04/2000

Residence: 38000 Grenoble - FRANCE

Citizenship: French

Post Office Address: 11, rue Turenne F 38000 <sup>PRF</sup> Grenoble FRANCE

Full name of third inventor:

Inventor's Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Residence:

Citizenship:

Post Office Address:

Full name of fourth inventor:

Inventor's Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Residence:

Citizenship:

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159780



Attorney's Docket No. 3339-422

PATENT

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re: Chroboczek et al.  
Appl. No.: PCT/FR98/02344  
Filed: November 3, 1998  
For: TRANSFECTING PEPTIDE VECTOR,  
COMPOSITION CONTAINING SAME  
AND THEIR APPLICATIONS

Attn: DO/US

May 2, 2000

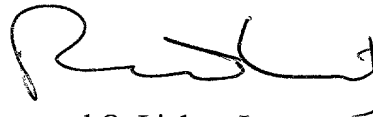
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SEQUENCE LISTING UNDER 37 CFR § 1.821(f)**

Box PCT  
Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

I hereby state that the content of the paper and computer readable copies of the Sequence Listing, submitted concurrently herewith in accordance with 37 CFR § 1.821(c) and (e), are the same.

Respectfully submitted,

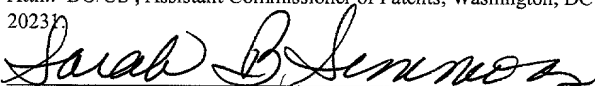


Raymond O. Linker, Jr.  
Attorney/Agent for Applicant  
Registration No. 26,419

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FENDER, Pascal  
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APPLICATIONS.

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